SOME OBSERVATIONS ON THE ASSIMILATION OF ATMOSPHERIC NITROGEN BY A FREE LIVING SOIL ORGANISM.—AZOTOBACTER CHROOCOCCUM OF BEIJERINCK.

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Some years before the appearance of Hellriegel and Wilfarth's work on the sources of nitrogen of leguminous plants, and while the part played by atmospheric nitrogen in the nutrition of crops was under active discussion, Berthelot was making some exact observations on the behaviour of uncropped soils towards the free element. He found that when 50 kilograms of air-dry arable soil were exposed to the air and rain in a vessel for seven months a great increase in the nitrogen content could be observed; the total nitrogen of the original soil had increased from 50 grams to 63, or a gain of over 25 per cent. after allowing for the small amount of combined nitrogen brought down by rain; in another experiment where the soil had first been washed free from nitrates, a gain of 46 per cent. of nitrogen was proved. In many other cases, however, the gain was only from 10–15 per cent. of the original nitrogen present in the soil. Gains have also been observed by many other observers but always much lower than those of Berthelot first mentioned. Berthelot was able to show that the gain of nitrogen in a soil kept in the dark was only half as great as in the same soil exposed to light; while, however, he could observe no increase in the nitrogen of his soil during the winter, Koch has found a considerable increase during that time in soil which was kept in a heap and shovelled over from time to time. Berthelot, observing that when the soil had been previously steamed at 100° no gain of nitrogen followed, concluded that soil organisms must be concerned in the fixation; he was unable, however, to separate by plate methods, a single

one which could assimilate free nitrogen in pure culture. In 1894 Winogradsky published his researches on a bacterium which could fix the free nitrogen of the air while growing in pure culture in a solution containing only dextrose and nitrogen-free salts; this organism he called *Clostridium pasteurianum*. It forms spores, is an anaerobe, and belongs to the group of butyric acid bacteria. The sugar is broken into carbonic acid, hydrogen, butyric acid, acetic acid and butyl alcohol, and for every gram of sugar consumed about 2 mgs. of nitrogen are assimilated from the air. In impure culture directly from the soil up to 3 mgs. of nitrogen are fixed for every gram of sugar decomposed.

During the period between Berthelot's work and the discovery of this organism the view had gained ground that algae growing on and near the surface of soil were able to fix nitrogen directly. In 1888 Frank had observed such a growth on sand exposed to light, and had found that the upper layer showed a considerable increase of nitrogen. In 1892 Schloesing and Laurent proved, both by determining the nitrogen fixed by a soil in a closed vessel, and by observing the diminution of the nitrogen gas in the enclosed air, that a soil exposed to light gains in nitrogen if algae are allowed to grow on the surface, and that the gain is confined to the upper few millimetres of depth. They did not however, employ a sterile soil nor pure cultures of algae. Kossowitsch working with pure cultures of two green algae found no fixation, but observed a considerable increase of soil nitrogen by growing them mixed with soil bacteria. Later Krüger and Schneidewind employing pure cultures of many other *Chlorophyceae* could also find no nitrogen fixation. Hellriegel and Déherain had also found very large increase in the nitrogen content of sand in pots when exposed to light, but always accompanied by a development of algae. In the light of such results the conclusion has been drawn that the algae cannot assimilate free nitrogen alone but only in concurrence with soil bacteria, the former producing carbohydrates which are used by the bacteria as a source of energy for the actual nitrogen fixation. Winogradsky's organism showed a very low efficiency of fixation for sugar consumed and was also strictly anaerobic, so that, as fixation in soil had been observed to be most abundant under aerobic conditions and often considerable in amount, the probability seemed strong that other and more active bacteria remained to be discovered. In 1901, Beijerinck was able to report upon a couple of closely allied organisms which gave a very abundant growth under highly aerobic conditions in solutions of carbohydrates containing no combined nitrogen; these he named

Azotobacter chroococcum and *Azotobacter agilis*. The former was widely distributed in cultivated soils and even in dune sand, the latter was only found in river and canal waters. *A. chroococcum* was obtained from soil by the method of 'elective culture,' so successfully employed by Winogradsky for the isolation of the nitrifying organisms. A few grams of soil were introduced into a solution of the composition:

Tap water	100
Mannite	2
Dipotassium phosphate	·02;

and incubated at a temperature of from 20°–30° either in diffused daylight or in the dark. After a few days a strong film formed on the liquid, consisting mainly of the organism, and by transferring a little to an agar made with the same solution and allowing isolated colonies to develop, a pure culture was obtained.

The mannite can be replaced by dextrose, levulose, cane sugar, dextrin, propionates, butyrates, and acetates, all of which are oxidised, according to Beijerinck, to carbonic acid and water. In mannite solution the growth is both active and fairly pure, whereas in dextrose more foreign organisms develop and cause acidity; and in propionates, butyrates, &c., the growth is very pure but less rapid and abundant. Beijerinck secured a fixation of nitrogen up to 7 mgs. for every gram of mannite or dextrose oxidised in the cultures directly seeded with soil, and similar amounts by inoculating repeatedly with the impure film. In pure culture the *Azotobacter* refused to grow and fix nitrogen in two cases out of four and in the other two fixed 2·70 and 5·50 mgs. N per litre of culture, or only ·13 and ·27 mgs. N for 1 gram of carbohydrate. By combining pure cultures of it with spore-forming butyric organisms (*Granulobacter* sp.) or with non-spore formers separated from the films (*Radiobacter* and *Aerobacter*), he obtained better fixation, up to 5·9 mgs. N per gram sugar consumed. He made no attempt to get *Granulobacter* species alone to fix nitrogen and his attempts with *Radiobacter* and *Aerobacter* were also unsuccessful; nevertheless he asserts that all *Granulobacter* sp. can fix nitrogen and that *Azotobacter* cannot do so alone (although the above-mentioned results disprove the statement). Beijerinck supposes that in mixed cultures the *Granulobacters*, *Radiobacter* and *Aerobacter* gain the power to fix in the presence of *Azotobacter*, which grows itself at the expense of the combined nitrogen escaping from them into the solution.

Very soon, however, Gerlach and Vogel, Freudenreich and Koch, showed that *A. chroococcum* was able to fix considerable quantities of nitrogen in perfectly pure culture. Gerlach and Vogel found that old cultures of the *Azotobacterium* became weaker in their fixative power, and that the amount of N fixed per gram of sugar consumed depended both upon aeration and the concentration of the sugar; 12 grams per litre was found most favourable, giving a fixation of 9–10 mgs. N per gram of sugar oxidised.

Freudenreich has found about the same ratio for cultures on gypsum, and Koch for those on agar. Gerlach and Vogel have proved that the organism cannot grow without phosphoric acid and calcium, but can do without magnesium and potassium. As regards potassium their results are not conclusive, since while as much N was fixed in 20 days without it as in a culture to which it was added, after 40 days there had been no further fixation in the former solution, but it had nearly doubled in the latter, suggesting that the traces of potash in the chemicals and dissolved from the glass during sterilisation had been enough to carry on development for a time only.

The Author's Observations.

In order to gain the first or impure culture of *Azotobacter* from soil a solution of the following composition has been used:

Mannite	12 or 20 g.
Monopotassium phosphate ...	·2 g.
Magnesium sulphate cryst. ...	·2 g.
Sodium chloride	·2 g.
Calcium sulphate	·1 g.
Distilled water	1 litre.

The phosphate was dissolved separately and rendered just alkaline to phenol-phthalein by the addition of N/10 sodium hydrate. 75 c.c. or 100 c.c. of the solution was then put into Erlenmeyer flasks of 250 or 300 c.c. capacity and sterilised in an autoclave at 130° for 15 minutes. Each flask then received ·5 g. of calcium carbonate (precipitated) and 1 g. of soil. Incubation was maintained at about 30°. After two days the solution becomes somewhat clouded and gas bubbles rise from the bottom of the flask; on the third day a gelatinous colourless ring forms on the glass along the edge of the liquid and from it a film rapidly extends and covers the whole surface of the liquid; this is at first white, opaque, dry, often very reticulate, and it may or may not be filled with gas bubbles. After a couple of days

it becomes yellow, later brown and finally almost black. The last state is arrived at in from 7–10 days, and the liquid beneath is usually quite clear. By the tenth day the mannite has usually disappeared, sometimes even at the end of a week. If allowed to remain after the disappearance of the carbohydrate the solution begins to cloud again and the film breaks up and falls to the floor of the flask; at the same time a marked putrefactive odour is perceived, due to the attack on the proteid of dead *Azotobacter* cells by putrefactive bacteria. If it is proposed to determine the amount of nitrogen fixed this latter stage must not be allowed to proceed since it results in loss of nitrogen as ammonia and other volatile compounds. The nitrogen has been determined by transferring the whole contents of the flasks to Jena glass combustion flasks, acidulating with sulphuric acid and evaporating to a small volume before adding the concentrated acid. As a rule there is an odour of butyric acid and fruit ether from the culture, the acid odour being most marked during concentration. When the butyric odour is much in evidence *Clostridia* can be found in flocks overlying the carbonate and soil on the bottom of the flask but rarely in the surface film, and this is accompanied by a relatively poor fixation of nitrogen. This occurs more often in cultures to which no calcium carbonate has been added. To obtain pure cultures a little young film is well shaken in sterilised distilled water and a drop spread over mannite agar in a petri dish. The agar must first be well washed for some days with frequent changes of cold water after cutting up, then partly dried with the aid of the filter pump, and finally dissolved in the culture solution mentioned above in the proportion of 1½–2 g. per litre. If unwashed agar is used too many colonies of foreign organisms develop before the *Azotobacter* and fuse with its colonies. Even in purified mannite agar transparent watery colonies of fluorescent bacteria appear in one day, and the *Azotobacter* colonies which appear about a day later often grow over them or fuse with them so that one cannot depend on getting a pure culture by inoculating off the first plate. In a second plating one can generally find colonies of *Azotobacter* isolated enough to secure a pure culture after transferring to an agar slant.

Appearance of Azotobacter chroococcum.

In the young impure films on solutions inoculated with Rothamsted soil the organism appears at first mainly in oval forms, often united in pairs, but also as cocci and diplococci, the former measuring 4–5 μ in length by 3 μ in width and the latter 2·5–4 μ in diameter for the

individual cell. If stained with iodine in potassium iodide many only assume a golden yellow colour, others are more brown and many of the cocci are of a deep red-brown. This latter colouration has been shown by Heinze to be due to glycogen similar to that which occurs in yeast cells. In old cultures the cells are practically all stained red-brown by iodine. If a little of a very young film is examined in a hanging drop of culture liquid, a cell here and there will be observed to suddenly become sluggishly motile and slowly cross the field of vision. Zettnow has found that the motion is due to a bundle of cilia at one pole. On mannite agar the colonies first appear as milky white glistening drops, round and convex, which under a low magnification show a coarsely granular structure extending to the margin. The colonies rapidly increase in size and after a week or more become brown at the centre, with concentric rings alternating dark and white to the circumference and darker streaks radiating from the centre outwards. In old cultures, where the agar has partly dried up, the cells are often united in Sarcina-like packets, the cell walls are much swollen, and the contents are aggregated to a small ball at the centre; at the same time giant cells both round and elongated can be seen filled with oil drops, and often a number of involution forms drawn out into long threads with false septa.

Some cultures after repeated transference on agar slants lose the power of turning dark with age and do not again recover it. On peptone beef extract agar the organism gives hardly any growth, but grows well when 1 per cent. dextrose is added, though the power of forming glycogen seems to be lost on this medium. In broth containing no added sugar there is practically no growth, the medium remaining clear with the formation of a slight sediment; if dextrose is added there is abundant multiplication at the surface of the liquid and a considerable sediment. To test the purity of cultures, seeding has been made into broth, and plates have been prepared with peptone beef extract agar with dextrose added; if the culture was pure the only colonies appearing on the peptone dextrose medium were those of *Azotobacter*.

Fixation of nitrogen by pure cultures.

In some early experiments with pure cultures of the Rothamsted organism great difficulty was found in getting it to grow in solutions containing either mannite or dextrose and prepared either with tap water or distilled water. After five weeks there was very slight

multiplication, and the quantity of nitrogen fixed was infinitesimal, although the cultures used had been freshly isolated, and were growing vigorously on the solid medium. Later, in order to secure an abundant inoculation the following method was adopted: 50 c.c. portions of dextrose and mannite culture solutions containing 12 g. to the litre and made up with tap water, received ·5 g. calcium carbonate, and were then sterilised. Flasks of 250 c.c. capacity received 10 c.c. of dextrose or mannite agar, and were then sterilised. The culture of *Azotobacter* was rubbed over the surface of this agar and allowed to develop for two or three days at 30°. When the growth had become abundant 50 c.c. of a sterile liquid medium, made up with tap water containing 12 g. of either mannite or dextrose in the litre and 1 per cent. calcium carbonate, was poured on to it and the flasks incubated at 30°. As a result of this method of inoculation the organism multiplied abundantly and formed a film on the surface of the liquid. Controls prepared in the same way but not inoculated were incubated with the cultures.

The results, with these pure cultures of organisms from three different places are given in the following table:

TABLE I.

Nitrogen fixed in milligrams for 1 *gram of carbohydrate.*

Origin	Carbon source	Time	1st plating	2nd plating	3rd plating
		days	mgs.	mgs.	mgs.
Mombasa, E. Africa	mannite	20	7·30	5·12	7·24, 7·82
	glucose	20	6·73	6·18	7·85
Cairo, Egypt	mannite	20	7·64	—	—
	glucose	20	5·73	—	—
Rothamsted	mannite	40	4·91	3·77	—
	glucose	40	4·62	3·57	—

A further reference will be made to the organisms from Africa under the final heading.

To test whether the mannite had disappeared, a drop or two of the solution was evaporated to dryness on a watch-glass and examined under the microscope for the characteristic needles; for dextrose by running a little of the culture into a boiling solution of methylene blue in caustic potash and observing whether the colour was discharged. Both tests were employed by Beijerinck.

Fixation was considerable in all cases and with the Mombasa *Azotobacter* was not weakened after three successive platings. The Rothamsted organism developed much more slowly, and gave a lower yield of nitrogen, thus showing a marked diminution in power to fix after the second plating.

Fixation of nitrogen by impure cultures.

Soil from many of the Rothamsted plots has been tested for the presence of *Azotobacter*, and the amount of nitrogen fixed in solutions seeded with it has been determined.

The following experiment brings out the influence of aeration on nitrogen fixation. The solution used contained 20 g. of mannite in the litre. The cultures were incubated at 30° for ten days, and each received 1 g. of soil as inoculation, and ·5 g. $CaCO_3$.

Soil	Solution	Total N fixed	N fixed per 1 g. mannite
		mgs.	mgs.
Barnfield 1 c.	100 c.c.	11·9	5·95
,,	50 c.c.	9·2	9·20
4 c.	100 c.c.	9·1	4·55
,,	50 c.c.	8·8	8·80

The flasks used were of 250 c.c. capacity in which 50 c.c. of solution only forms a thin and fully aerated layer.

It is evident that the superior aeration with only 50 c.c. of solution caused a much greater fixation of nitrogen per gram of mannite oxidised. A stronger film of *Azotobacter* also developed.

In the following table will be seen the amounts of nitrogen fixed both where *Azotobacter* developed and where no trace of it could be found.

TABLE II.

50 c.c. solution, 2 g. soil and incubated 30 days at 25°–30°.

Soil	N fixed for 1 g. mannite	*Azotobacter*
	mgs.	
Broadbalk Wilderness + $CaCO_3$	8·80	abundant
,, ,, ,, alone.....	7·30	,,
Soil from drain gauge + $CaCO_3$	6·60	fairly abundant
,, ,, ,, alone.....	5·10	,,
Geescroft Wilderness + $CaCO_3$	3·53	absent
,, ,, ,, alone	2·60	,,
Harpenden Common + $CaCO_3$	3·73	,,
,, ,, ,, alone.....	2·90	,,
Park soils		
Plot 1, unlimed.....	3·94 and 1·15	,,
,, limed.....	3·66 and 3·80	,,
Plot 4·2 unlimed	3·59	,,
,, limed	3·35	,,
Plot 9 unlimed	3·21	,,
Average fixation	6·95	*Azotobacter* present
,, ,, ...	3·22	,, absent

In presence of *Azotobacter* the average yield of nitrogen was more than doubled. When *Azotobacter* was absent there was a long continued production of gas and a foamy very thin film, a strong odour of butyric acid and flocks of *Clostridia* on the bottom of the flasks. It is evident then that fixation of nitrogen takes place even in the absence of *Azotobacter*, but is always very low in amount, approaching the yield found by Winogradsky for his *Clostridium*. It may be added that quite recently Thiele has obtained from soil several *Clostridia*, distinct from *C. pasteurianum* and capable of fixing up to 3 mgs. of nitrogen for every gram of dextrose fermented. Some of them also ferment mannite.

In all experiments with Rothamsted arable soils where fixation of nitrogen occurred *Azotobacter* was abundant in the film on the cultures.

The following are the best results obtained with pure and crude cultures of the organism.

Pure cultures	Mixed soil cultures
mgs. N fixed for 1 g. mannite oxidised	mgs. N fixed for 1 g. mannite oxidised
7·30	9·2
7·64	8·8
7·24	8·8
7·82	9·2
Average 7·50	9·22
	9·53
	Average. 9·12

It seems that in some way concurrence with other bacteria in the primary cultures acts favourably on fixation, provided the reaction remains neutral or alkaline; free acid (as in cultures without calcium carbonate which give a butyric fermentation) seriously affects the growth of *Azotobacter*.

Conditions favourable for fixation of Nitrogen.

1. *Aeration.*

The value of an abundant supply of air is well shown in the results of Table I.; in this case a solution of only half the depth with a similar extent of surface gave nearly twice as much fixation per gram of mannite oxidised. The more rapid growth on solid media as against liquids is also a proof of the great requirements of the organism for oxygen. In shallow cultures the injurious butyric fermentation, which is more or less anaerobic, is partly or wholly suppressed.

2. *Presence of a base.*

In Table II. the amount of fixation in the presence and absence of calcium carbonate is shown. It will be seen that where *Azotobacter* was present the addition of base increased fixation. In the following experiment the amount of nitrogen gained was determined at the end of ten days in 100 c.c. solutions containing 2 g. of mannite, with and without the addition of ·5 g. calcium carbonate. The solutions were inoculated with 1 g. of soil and incubation was conducted at 29°–30°.

Soil	Depth	$CaCO_3$ added	No addition of $CaCO_3$
		Nitrogen fixed mgs.	Nitrogen fixed mgs.
Little Hoos	10 cms.	13·13	5·33
,, ,, ...	20 cms.	9·78	4·79
,, ,, ...	30 cms.	5·21	3·98
Agdell, unmanured ...	10 cms.	9·36	4·61
,, full manured...	10 cms.	5·86	0·00

The figures show the total amount of fixation, the mannite not having been all oxidised in most cases. The *Azotobacter* has developed more rapidly in the series with calcium carbonate, which has secured growth and fixation in all cases, whereas both were wanting in one of the solutions receiving no carbonate.

In the following experiment an attempt was made to judge of the distribution of *Azotobacter* in several plots on Agdell Field at Rothamsted, where a four course rotation has been maintained with and without manures for over 50 years. One half of the plots had been left fallow every fourth year, and the other half seeded with beans or clover. The manures, all applied to the root crop, were on one-third of the land, phosphates and potash only; on another third, the same mineral manures and nitrogen; and on the remaining third, no manure. At some time before the experiments began the no manure, and about half the mineral plots, had been heavily chalked in the past, but the other half of the mineral plots, and the minerals with nitrogen plots, were unlimed, containing at present only a trace of carbonate. The soil was taken at a depth of 15 cms., and 1 g. was seeded into 75 c.c. solutions containing 12 g. mannite to the litre. To some solutions ·5 g. calcium carbonate was added, to others the same amount of magnesium carbonate, but most were left neutral. The flasks were incubated at 30° degrees for twelve days. Early in May, 1906, when the samples were taken, one half of each plot was under alsike clover, and the other half in fallow.

TABLE III.

Plot	N fixed for 1 g. mannite			Film appeared after		
	Neutral	With $CaCO_3$	With $MgCO_3$	days	days	days
	mgs.	mgs.	mgs.	Neutral	$CaCO_3$	$MgCO_3$
Minerals-fallow, chalked end	6·50	—	—	3½	—	—
unchalked end ...	4·75	6·80	8·00	4	4	6
Minerals-clover, chalked	5·80	—	—	5–6	—	—
unchalked	0·00	4·80	9·22	none	5	6
Unmanured-fallow, chalked	6·90	—	—	5–6	—	—
Unmanured-clover, chalked	4·30	—	—	7	—	—
Full manured-fallow, unchalked	4·20 & 0·00	5·80	9·53	4 & none	5	8
Full manured-clover unchalked	0·00	3·78	0·00	none	8	none

All the cultures were made in duplicate, and the figures represent the mean fixation for the pairs. The figures for the neutral solutions show that *Azotobacter* developed in every case from the fallow soils whether containing carbonate of lime or not; two of the clover soil inoculations failed to show any growth of *Azotobacter* and were not analysed, the average fixation from the fallow being 5·06, and from the clover land only 2·52. The chalked parts always gave *Azotobacter*, but there were three failures from the unchalked land, the average fixations being 5·87 and 1·71 respectively. Where there was failure to develop *Azotobacter* in the neutral solution, addition of calcium carbonate secured a film in each case. From the results with the neutral solutions it may be concluded that *Azotobacter* is more abundant in fallow than under clover, and much more abundant in soils well provided with carbonate of lime than in others where the latter is almost absent. The results shown in Table II. present a striking confirmation of the latter conclusion. In the three cases where *Azotobacter* developed and nitrogen fixation was large the soils contained abundance of calcium carbonate. In every case where *Azotobacter* failed to appear, the soils contained only a trace of carbonate, and even addition of carbonate to the solutions was ineffectual. All the latter soils were acid to litmus.

Magnesium carbonate as base.

As shown in Table III., an addition of magnesium carbonate in place of calcium carbonate caused a very large fixation of nitrogen in three cultures out of four, but the film developed much later. The average fixation with magnesium carbonate was 8·92 mgs., with calcium carbonate 5·80 mgs. The film developed with magnesium carbonate in 6·6 days and with calcium carbonate in 4·6 days. With magnesium carbonate there was 50 per cent. more nitrogen fixed, and a delay of two days in development. Examination of the cultures showed that the film with magnesium carbonate contained far less foreign organisms, and that whereas with calcium carbonate there was a butyric or fruit ether odour, the cultures with magnesium carbonate were quite odourless, even during concentration with acid.

A special experiment was therefore made to compare the influence of the carbonates of magnesium and calcium upon fixation and growth.

To three parallel solutions of 75 c.c. containing 12 g. mannite to the litre, ·5 g. calcium carbonate was given, to another set ·5 g. magnesium carbonate, to a third ·25 g. of each carbonate, and another was left neutral. All were inoculated with ·5 g. soil and incubated for 14 days

at 29°–30°. The amount of nitrogen fixed is expressed as mgs. per gram mannite.

	Neutrals	$CaCO_3$ series	$MgCO_3$ series	$CaCO_3 + MgCO_3$
1.	5·75	7·8	8·12	8·71
2.	4·98	7·15	7·62	7·08
3.	—	6·70	8·25	—
Average	5·36	7·22	8·00	7·89
Film appeared in days	4–5	3–4	8–10	6

During concentration the neutral cultures developed a strongly acid odour, those with calcium carbonate a weaker one, and those with magnesium carbonate, alone or mixed with calcium carbonate, gave no odour. When magnesium carbonate was present, development was greatly delayed, but the yield of nitrogen was again larger, though not to so marked an extent as in the earlier experiment. In pure culture *Azotobacter* gives rise to no acidity, either in solutions or on agar. One must conclude, therefore, that magnesium carbonate not only neutralises more effectually than calcium carbonate any trace of acidity due to foreign organisms in the early stages of culture, but also prevents butyric fermentation, but at first it inhibits the growth of *Azotobacter* itself.

Nitrogen fixation by soils taken at different depths.

Only one experiment has been made in this connexion. The writer has described in another place a method for obtaining and preparing an average soil sample[1]. The soil was taken from a fallow plot at depths of 10, 20 and 30 cms., and equal quantities were seeded into 100 c.c. portions of a culture solution containing 20 g. mannite in the litre. ·5 g. calcium carbonate was added, and incubation maintained at 29°–30°.

Little Hoos fallow soil.

Depth	7 days	10 days	13 days
	mgs.	mgs.	mgs.
10 cms.	6·84	13·13	12·15
20 cms.	3·77	9·78	11·03
30 cms.	2·93	5·21	10·20

[1] "The comparative nitrifying power of soils," *Trans. Chem. Soc.* 1904, vol. LXXXV., p. 1158.

The figures show the total nitrogen gained by the cultures during the times stated. The greatest contrast was after ten days. During this time the mannite had all been oxidised in the cultures inoculated with soil at 10 cms. depth, so that no further gain of nitrogen took place during the remaining three days. With soil from 20 cms. depth most of the sugar had gone after ten days, but with that from 30 cms. not till after thirteen days. The results show that *Azotobacter* is most abundantly present in soil near the surface, and falls off in amount with increasing depth. It is also evident that fixation does not occur uniformly over the period of active oxidation, but increases rapidly up to a point, and then becomes slower, corresponding with the greatly lessened concentration of carbohydrate.

Azotobacter from African soils.

It had been observed that, when a little of a soil which gave a growth of the organism in solutions was rubbed over a mannite agar plate, the characteristic colonies develop in two or three days, together with many other forms which, however, soon cease to grow, while the *Azotobacter* colonies spread rapidly and soon begin to darken.

Mr Hall took with him to South Africa in 1905 a number of such plates, and inoculated them with fresh soil from several parts of the "high veldt," and one with a tropical cultivated soil at Mombasa, in E. Africa. On examination it was found that *Azotobacter* had developed in only two cases, giving a gelatinous nearly black growth, which covered the greater part of the plates. An attempt was made to get the organism in pure culture by growing in nutritive solutions. The material was very impure and contained many amoebae and infusoria, which live largely on the cells of *Azotobacter*. The Mombasa organism was obtained pure by plate inoculations from a film which had slowly formed on a solution of sodium butyrate. The other organism, from a "vlei" soil near Lichtenburg in the Transvaal, did not give even a relatively pure growth, and could not be isolated. The film of the Mombasa organism on the butyrate was white, dry and brittle, and was remarkable for the very large size of the round cells, 5–6 μ diameter, which were rarely united. In pure culture, this Mombasa organism differs from all others examined by the production of a soluble pigment, at first greenish-blue, and later, light yellow in tint, which not only colours the growth on agar, but diffuses into both the solid and liquid media. From the first, the pure culture had no power of turning brown with age. On mannite and

glucose agar the growth is very rapid and more fluid than that given by Rothamsted organisms, and under the microscope the cells are isolated, very small, and almost invariably round; the diameter varying from 2–2½ μ. Iodine in potassium iodide only stains it yellow, so that the power of forming glycogen seems to have been lost. The formation of the pigment is markedly favoured by the presence of calcium carbonate both in solid and liquid media. On one mannite agar plate, where carbonate had been first added but had accumulated at one end, the growth above the carbonate alone produced pigment, which was quite absent on the portion where carbonate was wanting. Calcium carbonate seems to have a specific action on the production of pigment, the colour of which is at once discharged by mineral acids.

The very active fixation of nitrogen by pure cultures of the Mombasa organism even after repeated transference, has already been referred to (see Table I.). Its behaviour towards media containing organic nitrogen is similar to that described for the Rothamsted organism. In a solution containing dextrose and ·02 per cent. nitrogen as calcium nitrate growth is abundant, and the nitrate is very slowly and only partly converted to ammonia.

The Cairo organism was isolated from a sample of fresh soil collected at Ghezirah by Mr R. Aladjem of the Agricultural Experiment Station there. This soil showed a markedly alkaline reaction to litmus paper, due to alkaline carbonate, but the bulk of the carbonate present was calcium carbonate. The crude cultures inoculated with soil were remarkable for the speed with which the film developed and the mannite disappeared, fixation being complete in five days, by which time the film was quite black. The fixation was also identical in amount from cultures with varying amounts of solution, and with and without added carbonate. Even after ten days there was no putrefactive odour, and no trace of a butyric fermentation could be detected. The fixation was in every case 9·2 mgs. nitrogen for 1 gram mannite oxidised.

In pure culture this organism is in every way similar to that from Rothamsted, with the single exception that fixation is with it more rapid and greater (see Table I.) in amount. The power of turning brown, and finally black with age, has been preserved in the pure cultures.

Although the vlei organism from S. Africa was not isolated, it produced a pigment quite similar to the Mombasa form in impure culture, and must certainly be classed with it as one variety. As descriptions by continental authors agree fully with the Rothamsted and Cairo type, one must conclude that there is one variety common to

Europe and extending to Egypt, and another quite distinct found in East and South Africa.

The organism separated by Beijerinck from waters and named *A. agilis*, is a larger form which is very actively motile in young liquid cultures, and also forms a diffusible pigment, green in solutions of salts of organic acids and reddish-violet in the presence of carbohydrates.

Effect of desiccation on Azotobacter chroococcum.

No spores are formed, yet the organism can resist drying up in the air for a long time. A soil, which was known to contain the organism in the fresh condition, after being kept air-dry in bottle for a year, still yielded an abundant growth after inoculation into a mannite solution. Old cultures of the organism on agar which had dried down to a leathery consistency, after many months still showed abundant growth after pouring a fresh culture solution over them. It is evident then, that the organism can be freely distributed in dust by the wind.

General Remarks.

Several observers have been struck by the resemblance of the organism to some of the unicellular Algae. Beijerinck believes it to be closely related to Winogradsky's *Chromatium*, while Benecke and Keutner consider it a colourless form of one of the *Cyanophyceae* namely *Aphanocapsa*, but no one has yet been able to induce it to produce chlorophyll by cultivation in light. Attempts have been made to bring about nitrogen fixation by seeding pure cultures into sterilised and unsterilised soil, but as yet without success. Similar experiments are now in progress at Rothamsted. A. D. Hall has recently reported on two cases of considerable nitrogen increase in Rothamsted soils allowed to run wild for many years. In one, showing the greater increase, *Azotobacter* was abundantly present, in the other it could not be found, but butyric organisms were present (see Table II.).

Some references to the more important papers dealing with the subject are given below. Where the *Centralblatt für Bakteriologie* is cited the Second Part is always meant.

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